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Report

Dosage Compensation and Demasculinization of X Chromosomes in *Drosophila*

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Summary

The X chromosome of *Drosophila* shows a deficiency of genes with male-biased expression [1–4], whereas mammalian X chromosomes are enriched for spermatogenesis genes expressed premeiosis and multicopy testis genes [5, 6]. Meiotic X-inactivation and sexual antagonism can only partly account for these patterns. Here, we show that dosage compensation (DC) in *Drosophila* may contribute substantially to the depletion of male genes on the X. To equalize expression between X-linked and autosomal genes in the two sexes, male *Drosophila* hypertranscribe their single X, whereas female mammals silence one of their two X chromosomes. We combine fine-scale mapping data of dosage compensated regions with genome-wide expression profiles and show that most male-biased genes on the *D. melanogaster* X are located outside dosage compensated regions. Additionally, X-linked genes that have newly acquired male-biased expression in *D. melanogaster* are less likely to be dosage compensated, and parental X-linked genes that gave rise to an autosomal male-biased retrocopy are more likely located within compensated regions. This suggests that DC contributes to the observed demasculinization of X chromosomes in *Drosophila*, both by limiting the emergence of male-biased expression patterns of existing X genes, and by contributing to gene trafficking of male genes off the X.

Results and Discussion

In many animals with separate sexes, a large fraction of the genome shows sex-biased gene expression [7]. In *Drosophila*, for example, between 30% and 60% of the transcriptome is expressed differently in males and females (sex-biased genes) [4, 8, 9]. Genes with sex-biased expression often show a nonrandom genomic distribution, with male-biased genes being significantly depleted on the *Drosophila* X chromosome [1, 2, 4].

Two main models have been proposed to explain the observed deficiency of male-biased genes on the X [10–12]. The X of several taxa is transcriptionally inactivated early in spermatogenesis (meiotic X inactivation [11, 12]), implying that the X is a disfavored location for genes required during spermatogenesis, and X-linked copies of such genes will be selected against in favor of autosomal copies [1, 3]. Although X inactivation certainly plays a role to explain the depletion of testis-biased X-linked genes, it cannot account for the observed deficiency of X-linked male-biased genes in somatic tissues [2, 7, 13]. Sexually antagonistic selection can also contribute to observed patterns of X-chromosomal gene content [7, 13, 14]. Specifically, mutations that are beneficial to one sex while deleterious to the other (sexually antagonistic mutations) can accumulate differently on sex chromosomes [10]. Because of hemizygosity of the X in *Drosophila* males, recessive male-beneficial mutations will accumulate more rapidly on the X relative to autosomes whereas dominant female-beneficial mutations are expected to fix more easily on the X because of its female-biased transmission [10]. If sexually antagonistic mutations are more often dominant, this could explain the deficiency of male-biased genes on the X of *Drosophila* [1].

Here, we provide evidence for a third force contributing to the depletion of male-biased genes [15]: hypertranscription of the single X chromosome in *Drosophila* males (i.e., dosage compensation [16, 17]). To equalize expression levels between autosomal and X-linked genes, *Drosophila* males recruit an RNA/protein complex (termed the MSL complex) to their single X chromosome, which induces acetylation of histone H4 [17, 18]. This results in a global change of chromatin structure, facilitating increased rates of transcription of X-linked genes in males [19]. Recent high-resolution mapping experiments support a two-step model of MSL recruitment to the X chromosome of *Drosophila* [20–23] (see Figure 1A). The MSL complex is thought to first target over 100 chromatin entry sites (termed high-affinity sites [HAS]) containing specific MSL recognition elements on the X in males [20]. After this initial, sequence-specific recognition step, local spreading from entry sites in *cis* along the chromosome leads to MSL binding to the majority of active genes on the X [21, 22, 24–27].

The mechanism of dosage compensation in *Drosophila* males could contribute to the observed deficiency of male-biased X-linked genes through direct or indirect effects. First, mechanistic or functional constraint could actively limit further upregulation of already hypertranscribed X-linked genes in males [15]. Specifically, the modified chromatin structure of the X may directly interfere with subsequent transcriptional modification of X-linked genes in males [28], or transcription rates may have reached an upper limit on the hyperactive X [15]. Second, the *Drosophila* X chromosome may undergo incomplete dosage compensation, with many genes on the haploid male X showing little or no upregulation. Lack of dosage compensation would indirectly result in a deficiency of genes with male-biased expression on the X relative to autosomes. The two-step model of dosage compensation makes several predictions that allow us to distinguish between these two hypotheses (Figure 1B). If the deficiency of male-biased genes in *Drosophila* is a consequence of the dosage compensation machinery directly interfering with further upregulation of X-linked genes, genes with male-biased expression should mainly be found outside dosage compensated regions. In particular, (1) genes with male-biased expression should, on average, be further away from the nearest HAS; (2) genes further away from a HAS should exhibit more male-biased gene expression; (3) genes with male-biased gene expression should be dosage compensated less frequently (less likely be bound by the MSL complex) than genes with unbiased or female-biased expression. Alternatively, if the depletion of male-biased genes indirectly results from incomplete dosage compensation, male-biased genes should mainly reside in

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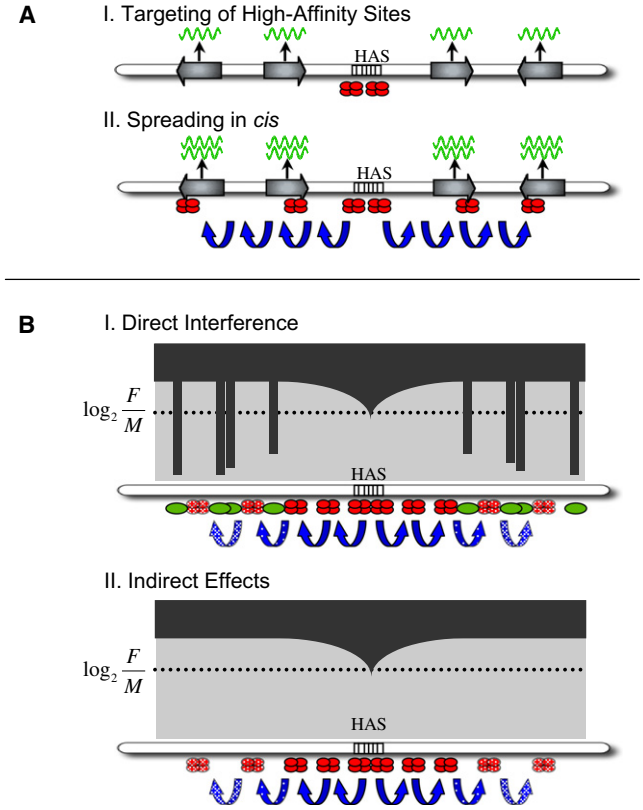

Figure 1. Models of Dosage Compensation and Sex-Biased Expression in *Drosophila*

(A) The two-step model of MSL targeting to the X chromosome. I: The MSL complex (red circles) targets specific HAS on the X chromosome in a sequence-dependent manner. HAS represent a subset of the MSL-bound regions in wild-type *Drosophila* that also recruit the MSL complex under more stringent conditions (such as when inserted into an autosome, or when integral subunits of the MSL complex are missing). II: After initial targeting, the MSL complex spreads along in *cis* from the entry sites (shown by blue arrows), and predominantly binds to the 3′ end of actively transcribed genes. MSL binding causes acetylation at histone H4 and results in a global change of the chromatin structure, facilitating a two-fold transcriptional upregulation of X-linked genes in males (green lines).

(B) Models of sex-biased expression versus dosage compensation in *Drosophila*. I: Direct interference of the dosage compensation machinery with male-biased expression. Binding of the dosage compensation complex, changes in chromatin structure, and global hypertranscription of X-linked genes may interfere with subsequent transcriptional modifications and upregulation of X genes in males (green circles). Genes further away from a HAS (or those not bound by the MSL complex) are more likely to be upregulated in males. II: Indirect effects of dosage compensation on sex-biased gene expression. Genes further from a HAS will less likely be compensated in males, resulting in female-biased expression for genes further away from a HAS.

dosage compensated regions, and we would expect to see opposite patterns (i.e., male-biased genes should be closer to the nearest HAS; genes closer to a HAS should be more male biased in expression; and genes with male-biased expression should more often be MSL bound). Finally, if dosage compensation has no influence on patterns of male-biased expression, sex-biased genes should be distributed randomly with regards to HAS and MSL-bound regions.

The Two-Step Model of Dosage Compensation

To test these predictions, we combine fine-scale mapping data of HAS and MSL-bound regions in *D. melanogaster* [20, 22, 23] with genome-wide data on sex-biased gene expression [29]. Recent high-resolution chromatin immunoprecipitation-sequencing (ChIP-seq) analysis has identified approximately 150 HAS along the *D. melanogaster* X [20, 23], and 53% (1132 out of 2142) of all X-linked genes studied were clearly bound by the MSL complex [21, 22, 24]. If the HAS indeed function as chromatin entry sites from which the MSL complex spreads, we expect that genes that are closer to such an entry site are more likely to be dosage compensated. We find that genes bound by the MSL complex in wild-type *Drosophila* are significantly closer to a HAS (median distance 12,507 bp) than unbound genes (median distance 49,311 bp; Wilcoxon two-sample test, $p < 0.0001$; Figure 2A). In addition, reduced X expression has been demonstrated in male-like tissue culture cells following RNAi knockdown of MSL2 [30], and genes bound by the MSL complex are more likely to be downregulated during RNAi treatment (defined by a 1.4-fold decrease or more) than those not bound (24% of bound genes are significantly downregulated, relative to 14% of genes not bound by MSL; $p < 0.01$). Thus, these data support the two-step model of MSL targeting to the X chromosome, with MSL binding directly resulting in upregulation of X-linked genes in *D. melanogaster* males.

Dosage Compensation and Sex-Biased Expression

To test for a link between dosage compensation and sex-biased expression, we categorized X-linked genes as male-biased, female-biased, and unbiased by using expression profiles from gonadectomized flies [1]. All analyses were repeated using expression profiles from whole adult flies or gonads to classify sex-biased expression, with similar results obtained (see Supplemental Results, available online). Patterns of MSL binding provide evidence that dosage compensation indeed is shaping sex-biased gene expression on the *Drosophila* X chromosome (Figure 2). Specifically, genes with male-biased expression are significantly further away from a HAS than female-biased or unbiased genes ($p = 5.406 \times 10^{-7}$ and $p = 4.462 \times 10^{-10}$, respectively, Wilcoxon two-sample tests, Figure 2A). Median distance to the closest HAS is 18,359 bp for female-biased genes and 17,453 bp for unbiased genes, but 46,543 bp for genes showing male-biased expression (Figure 2A). Additionally, we observe a significant positive correlation between the magnitude of male-biased expression (measured as the $\log_2$ male:female expression ratio) of individual X-linked genes and their distance to the closest HAS (Figure 2B, Kendall's $\tau = 0.109$, $p = 3.46 \times 10^{-8}$). Thus, contrary to incomplete dosage compensation indirectly resulting in a deficiency of male-biased genes, this data suggests that it is easier to achieve male-biased expression away from compensated regions. Finally, if X-linked genes are classified as bound by the MSL complex or not bound, we observe a significant deficiency of genes with male-biased expression that are targeted by MSL (Figure 2C, $\chi^2 = 26.7$, degrees of freedom [d.f.] = 2, $p = 1.58 \times 10^{-6}$). Taken together, we find compelling support that dosage compensation is influencing patterns of sex-biased expression. Importantly, we find no evidence that a simple lack of dosage compensation is indirectly causing the observed deficiency of male-biased genes on the X. Instead, all our findings are consistent with dosage compensation actively limiting or interfering with the evolution of male-biased gene expression at the X chromosome of *Drosophila*.

Dosage Compensation and Demasculinization

X chromosomes of *Drosophila* are depleted of genes with male-biased expression [1, 2]. If dosage compensation

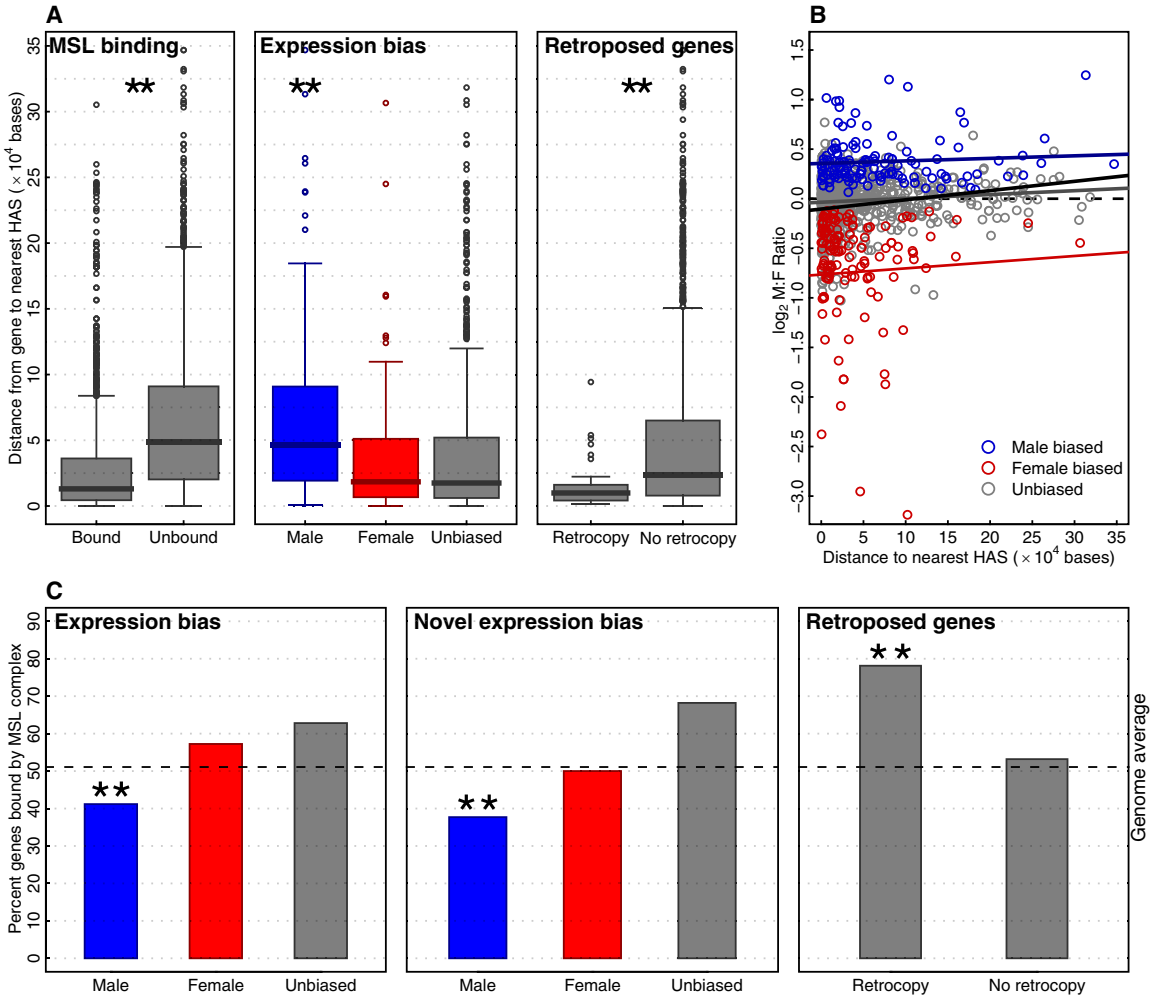

Figure 2. Patterns of Sex-Biased Expression versus Dosage Compensation in *Drosophila*

(A) Distance to nearest HAS for X-linked genes. X-linked genes are categorized according to their MSL-binding profile [20], expression bias [29], or patterns of retroposition [31]. Genes targeted by the MSL complex (bound) are significantly closer to a HAS than genes not bound (unbound). Genes with male-biased expression are significantly further away from a HAS compared to female-biased and unbiased genes. Parental genes that have a retrocopy in the genome are significantly closer to a HAS than genes not having a duplicate retrocopy.

(B) Sex-biased expression versus distance to HAS. Plot of gene expression sex ratio ($\log_2$ female/male ratio) for each X-linked gene in *D. melanogaster* against distance to its closest HAS. The lines are regression highlighting the trends: Red, female-biased genes; blue, male-biased genes; gray, unbiased genes. The black line describes the trend of all data.

(C) Fraction of dosage compensated genes. X-linked genes are categorized according to their expression bias [1], their change in sex-biased gene expression on the *D. melanogaster* lineage [29], or patterns of retroposition [31]. Genes with male-biased expression are significantly less likely to be bound by the MSL complex. Parental genes with a retrocopy are significantly more likely to be MSL bound than genes not having a retrocopy.

contributes to the deficiency of male-biased genes [1–4], we expect that X-linked genes not bound by the MSL complex contain a similar proportion of male-biased genes as autosomes, whereas MSL-bound genes are disproportionally deficient for genes with male-biased expression. Figure 3A plots the distribution of sex-biased genes across the different chromosomes, with X-linked genes further classified as either MSL bound or not. Genes with female-biased expression are randomly distributed across the genome, independent of their MSL-binding pattern (χ^2 = 6.9, d.f. = 4, $p > 0.05$). As shown previously, male-biased genes are underrepresented on the X relative to autosomes (χ^2 = 52.2, d.f. = 1, $p < 0.0001$). Strikingly, the observed deficiency appears to be almost entirely driven by a lack of MSL-bound male-biased genes. We find a highly significant underrepresentation of male-biased genes on the X that are targeted by the MSL complex (χ^2 = 81.0; d.f. = 1, $p < 0.0001$; Figure 3A), but not for male-biased genes not bound by MSL (χ^2 = 1.0; d.f. = 1, $p > 0.05$; Figure 3A). Specifically, ~8.1% of MSL-bound genes are male biased, but 15.1% of autosomal genes are classified as male biased, whereas X-linked genes not bound by the MSL complex show a proportion of male-biased genes (17.1%) similar to autosomes (see Figure 3A). Additionally, if global hypertranscription of the X in males limits further upregulation of individual genes, we not only expect to find fewer male-biased genes, but also the magnitude of sex-biased expression to be less for male-biased genes on the X relative to autosomal male-biased genes [15]. Indeed, absolute values of sex-biased expression ratios are similar for X-linked and autosomal female-biased genes (median $\log_2$ female:male expression ratio 0.496 versus 0.498, Wilcoxon two-sample test, $p > 0.5$, Figure 3B). However, sex-biased gene expression is

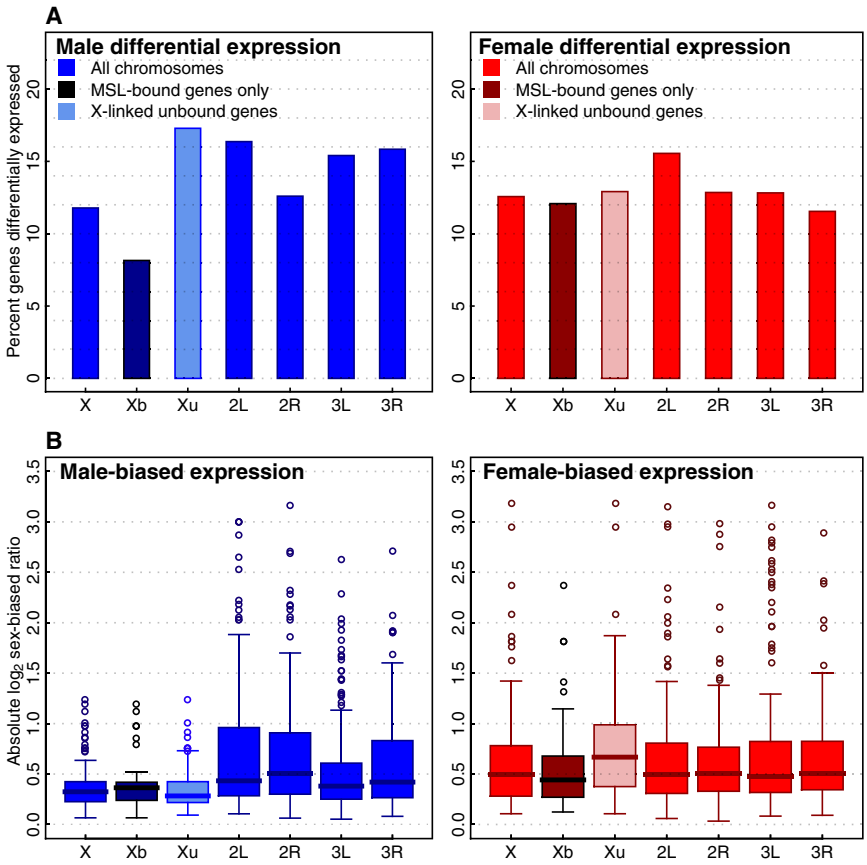

Figure 3. Distribution of Sex-Biased Genes in *Drosophila*

(A) Fraction of sex-biased genes across *Drosophila* chromosomes. The percentages of genes with male-biased (blue) or female-biased (red) expression are shown. X-linked genes are divided into those bound by the MSL complex (X_b) or not bound (X_u). Male-biased genes bound by the MSL complex are significantly underrepresented on the X.

(B) Sex-biased expression ratios across *Drosophila* chromosomes. Comparison of the extent of male- and female-biased gene expression ratios for each chromosome in *D. melanogaster*. Sex-biased gene expression is significantly lower for male-biased genes on the X relative to autosomes. The absolute value of the $\log_2$ expression ratio is plotted on the y axis. Bold horizontal bars are the median value, the box is the interquartile range, and the whiskers is the 95% confidence interval.

significantly lower for male-biased genes on the X relative to autosomes (median $\log_2$ male:female expression ratio 0.324 versus 0.420, Wilcoxon two-sample test, $p < 0.001$, Figure 3B). Thus, these results suggest that dosage compensation plays a significant role in explaining the demasculinization of the X chromosome in *Drosophila*. Consistent with the dosage compensation machinery limiting further sex-specific modifications of patterns of gene expression, genes with equal expression in the two sexes are more likely to be bound by the MSL complex than sex-biased genes (see Figure S4).

Dosage Compensation and Turnover in Sex-Biased Gene Expression

Sex-biased gene expression changes accumulate over time, with male-biased expression displaying a higher rate of turnover in the genus *Drosophila* than female-biased expression [4, 8, 9]. Extensive categorical changes in sex-bias class were reported between *D. melanogaster* and *D. simulans* (~12% of their orthologs show a categorical change in sex-biased expression), mostly between genes showing non-sex-biased expression and genes displaying modest sex-biased expression [29]. If dosage compensation is interfering with the evolution of de novo male-biased gene expression of existing genes on the X, we expect genes that have acquired male-biased expression less often to be bound by the MSL complex. To identify genes that have changed sex-biased expression patterns in the *D. melanogaster* lineage, we compared sex-biased gene expression profiles for orthologous genes from *D. melanogaster*, *D. simulans*, and *D. yakuba* [29]. Patterns of changes in sex-biased gene expression are consistent with dosage compensation limiting the acquisition of male-biased expression of X-linked genes. In particular, 989 genes are classified as male biased in *D. melanogaster* but not *D. simulans* or *D. yakuba* (11% of which are X linked), and 278 genes are classified as female biased specifically in *D. melanogaster* (13% of which are X linked). However, whereas 50% of newly female-biased X-linked genes are MSL bound (mirroring the chromosome-average pattern of MSL binding), only 37% of newly male-biased genes are bound by the MSL complex ($\chi^2 = 5.1$; d.f. = 1, $p < 0.02$; Figure 3C). Thus, the significant deficiency of genes in *D. melanogaster* with newly acquired male-biased expression bound by MSL provides independent evidence that dosage compensation is interfering with the evolution of male-biased gene expression on the X.

Dosage Compensation and Gene Trafficking

Comparative genomic studies in *Drosophila* have uncovered an excess of retrogenes that originate from the X chromosome and retropose to the autosomes, where they evolve male-biased expression [3, 31]. If dosage compensation is contributing to this exodus of genes by preventing the evolution of male-biased expression at their X chromosomal location, we expect parental X-linked copies of retroposed genes to reside closer to a HAS, on average, and more likely be bound by the MSL complex relative to X genes that have not produced a duplicate copy. A recent study utilizing whole-genome data from multiple *Drosophila* species identified over 90 retroposition events that gave rise to candidate functional genes in *D. melanogaster*, a third of which originated from parental genes located on the X [31]. Consistent with dosage compensation interfering with acquiring male-biased expression at these genes at their X location, parental genes giving rise to a retrocopy reside significantly closer to a HAS than nonparental genes (Figure 2A). Median distance to the closest HAS is 10,294 bp for parental genes with a retrotransposed duplicate, but 26,544 bp for genes without a retrocopy (Wilcoxon two-sample test, $p < 0.001$, Figure 2A). Additionally, parental genes that gave rise to a retroposed gene are significantly more likely to be MSL bound than nonparental genes (78% of parental

X-linked genes are MSL bound, compared to 51% genome average, $\chi^2 = 6.9$, d.f. = 1, $p < 0.01$, Figure 2C). Thus, patterns of retroposition provide further evidence that dosage compensation is limiting the evolution of male-biased expression, and suggests that dosage compensation significantly contributes to observed trafficking of male-biased genes off the X chromosome.

Conclusions

We find compelling evidence that dosage compensation influences patterns of sex-biased expression in *Drosophila*, and contributes to movement of male-biased genes off the X. Our analysis suggests that the deficiency of male-biased genes on the *Drosophila* X does not simply reflect a lack of dosage compensation at some genes but instead can partly be accounted for by dosage compensation directly interfering with further upregulation of MSL-bound, already hypertranscribed X-linked genes in males. The X chromosome in male *Drosophila* is encumbered by the MSL complex and its chromatin structure is modified globally, which may limit subsequent transcription factor binding or chromatin remodeling, and thus inhibit further transcriptional activation. Indeed, direct interference between chromatin remodeling complexes and the dosage compensation machinery has been reported in *Drosophila* [28]. Additionally, male-biased gene expression originates mainly by increasing transcription of nonbiased genes in males (rather than downregulation in females [15]), and higher expression levels may be harder to achieve on an already hypertranscribed chromosome. High-expression male-biased genes are located less often on the X than low-expression male-biased genes. This is expected if limits in rates of transcription prevent the accumulation of male-biased genes on the X, given that such limitations are less likely to affect genes that are transcribed only at low levels [15].

Not all organisms show a deficiency of male-biased genes on the X. In particular, mammalian X chromosomes are enriched for single-copy spermatogenesis genes that are expressed premeiosis [5, 6], and multicopy testis genes showing postmeiotic expression [32]. This difference in X-chromosomal gene content between taxa could result from fundamental differences in the mechanisms of dosage compensation [13, 33, 34]. Dosage compensation in mammals is achieved by first doubling global expression levels of the X in both sexes [35], followed by inactivation of one X in females [36]. The chromatin structure of the active X in mammals and baseline transcription rates of X-linked genes thus appear the same between the sexes (even though they might differ from average autosomal rates of transcription), therefore imposing no male-specific restrictions on the evolution of sex-biased expression patterns. Thus, the difference in X-chromosomal gene content between *Drosophila* and mammals—with a deficiency versus an accumulation of male-biased genes—may be understood in light of their vastly different dosage compensation mechanisms.

Experimental Procedures

Data

FlyBase *Drosophila melanogaster* release 5.5 was used to cross-reference the different data sets employed in this study. We used the physical location of each of the 150 HAS identified in [20] to calculate the distance from the 3′ end (the MSL complex preferentially binds to the 3′end of coding genes [20, 22]) to the nearest HAS of each X-linked gene obtained from FlyBase. Note that these 150 high-affinity sites were originally identified in msl3 mutant male embryos, and confirmed in MSL3-TAP male cell lines [20]. Using different experimental approaches and *Drosophila* lines to identify high-affinity sites (either by reducing levels of MSL-spreading factors by RNAi against MOF, MLE, or MSL3 or by lowering levels of crosslinking to reveal sites of more intimate contact of MSL proteins with DNA), reference [23] identified a highly overlapping set of HAS in *D. melanogaster*. In particular, 90 of the 130 X-chromosomal HAS identified in [23] perfectly overlap with the 150 HAS identified by [20]. We used the high-resolution ChIP-chip mapping data from [22] to classify X-linked genes as bound or unbound by the MSL complex; 1132 X genes of release 5.5 were classified as MSL bound, and 1010 were classified as unbound [22]. Sex-biased gene expression data for *D. melanogaster* were extracted from published global expression profiles of gonadectomized flies using species-specific microarrays [1]. The *D. melanogaster* expression data and platform descriptions can be found at the National Center for Biotechnology Information's (NCBI) Gene Expression Omnibus accession number GPL4629. We classified genes showing male-biased, female-biased, or unbiased expression by using the same criteria as in the database Sebida [37]. Briefly, gene expression data were inputted to Bayesian analysis of gene expression level (BAGEL) [38] to obtain p values and permutations of the data were input to BAGEL to obtain a 5% false discovery rate (FDR) cutoff. According to these criteria, 135 *D. melanogaster* X-linked genes were classified as male biased, 144 were female biased, and 864 were unbiased. In addition, we also performed our analysis on sex-biased gene expression versus dosage compensation with genes classified as male-biased, female-biased, and unbiased from whole adult flies and gonads [1]. The results of this analysis are shown in the Supplemental Information. To identify changes in patterns of sex-biased expression in the *D. melanogaster* lineage, we used global species-specific expression profiles from [29]. X-linked parental genes that gave rise to candidate functional retrogenes in *D. melanogaster* were taken from [31], which identified over 90 retroposition events by using whole-genome data for multiple *Drosophila* species; 31 events involved parental genes located on the X. We also performed Gene Ontology categorization versus MSL-binding patterns (Table S1) or sex-biased expression (Table S2). Datasets were integrated in R based on FBgn number. All the integrated data used are available in the Supplemental Information.

Statistical Analysis

To test for a difference in distance to the closest HAS for genes categorized according to their MSL-binding profile, their expression bias, or patterns of retrotransposition, we used Wilcoxon tests. We performed nonparametric Kendall τ rank correlations between distance to closest HAS and $\log_2$ male:female expression ratio for three groupings of X-linked expression data: male-biased, female-biased, and unbiased genes. A chi-square test was used to evaluate independence between MSL binding and expression bias or retroposition patterns, by comparing the observed number of genes showing binding for a given condition (i.e., male-biased, female-biased, unbiased or parental gene, nonparental gene) with the expected number based on the fraction of X-linked genes showing MSL binding. To test for heterogeneity in the chromosomal distribution of male-biased and female-biased transcripts, we calculated chi-square statistics, by comparing the observed number of genes showing differential expression (male- or female-biased) on each chromosome with the expected number based on the fraction of the genome contained on each arm. X-linked genes were further categorizes as bound by the MSL complex or unbound. To compare the distributions of sex-biased expression ratios between autosomal genes and X-linked genes, X-linked genes bound by the MSL complex, and X-linked genes not bound by the MSL complex, we used Wilcoxon tests. Bonferroni corrected p values are used to account for multiple testing.

Supplemental Information

Supplemental Information includes Supplemental Results, four figures, and one table and can be found with this article online at doi:10.1016/j.cub.2010.06.076.

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